APPENDIX B:

Carbohydrate-based microparticles as adjuvant for allergy vaccines: CBP coupled hypoallergenic Bet v 1 derivatives - a vaccine against birch pollen allergy

To study whether carbohydrate-based particles ("CBP") can be used as adjuvant in a vaccine against birch pollen allergy, two recombinant hypoallergenic derivatives of the major birch pollen allergen, Bet v 1 (i.e. Bet v 1F1 and Bet v 1F2) (1) were coupled to carbohydrate-based microparticles and further formulated to a vaccine containing equimolar amounts of the 2 fragments coupled to CBP (referred to as "F1+F2-CBP") . Blab/c mice were immunized with the vaccine and development of Bet v 1 specific IgG antibodies upon immunization (which is the desired outcome of the envisaged allergy vaccine) was monitored. Further, granulomatous reactions at the injection site after injection of F1+F2-CBP were compared to those induced by injection of an Alum-adsorbed mixture of Bet v 1F1 + F2 .

Coupling of antigens Bet v 1-F1 and Bet v 1-F2 to Sepharose beads (CBP):

The CBP used for the study were cross-linked agarose beads, pre-activated with N-hydroxysuccinimide to react with primary amino groups from the protein by forming a stable covalent bond. In this document the designation of "CBP" is used for these pre-activated beads.

The process for coupling of the antigens to CBP and preparation of bulk solutions of CBP-Bet v 1F1 and CBP-Bet v 1F2, included the following steps:

1. Preconditioning of the CBPs: 10 ml of the CBP suspension (in 100% Isopropanol, as delivered) were washed with 1mM HCl.
2. Preparation of the antigen to be coupled: An antigen solution containing 0,5 mg/ml Bet v 1F1 or Bet v 1F2 in 20mM $NaHCO_3$, pH 9.0 was prepared.
3. Coupling of the antigens to CBPs: The HCl washed CBP suspension was mixed with 20ml antigen solution and incubated for 2 hours at room temperature (20-24°C). After 2 hours the supernatant was removed.
4. Blocking of residual binding sites: The CBP-antigen conjugate was incubated with blocking buffer (0.1M Tris-HCl, pH 8.5) for 2 hours at room temperature (20-24°C). After 2 hours the blocking supernatant was removed.
5. Washing of the CBPs to remove non-coupled antigen: The CBP-antigen conjugate was washed in alternating cycles of 0.1M Tris-HCl, pH8.5 and 0.1M acetate buffer, 0.5M NaCl pH 4.5.

6. Preparation of a bulk suspension of CBP-Bet v 1F1 and CBP-Bet v 1F2: The CBP-antigen conjugate was washed for several times with sterile 0.9% NaCl. Finally, sterile 0.9% NaCl was added to the CBP-antigen conjugate to a total volume of 20 ml.
7. Filling and storage: Aliquots of the bulk suspension in 0,9% NaCl were transferred into sterile 2 mL polypropylene cryo-vials (Greiner, Cat. No. 122 279) closed with a screw-cap and stored at ≤ 8°C.

Steps 1, 5, and 6 of the coupling process are carried out in a 500ml glass filter funnel having a nominal pore size of 1,0-1,6µm and yield a mean particle diameter of 30 µm. Exchange of buffers/solutions and separation of buffers/ solutions from the CBPs is done by application of suction via a vacuum pump.

Steps 3 and 4 of the coupling procedure are carried out in sterile cell culture flasks on a shaker.

Formulation of a vaccine containing equimolar amounts of Bet v 1F1 and Bet v 1F2 (F1+F1-CBP):

The amount of Bet v 1F1 and Bet v 1F2 coupled on CBP-Bet v 1F1 and CBP-Bet v 1F2, respectively, was determined by measuring the protein concentration in the bulk conjugate suspensions using the QuantiPro BCA Assay Kit (Sigma Aldrich). The assay was performed according to the manufacturer's instructions leading to the following results:

CBP-Bet v 1F1	0.7 mg Bet v 1F1 per ml bulk conjugate suspension
CBP-Bet v 1F2	0.8 mg Bet v 1F2 per ml bulk conjugate suspension

The F1+F2-CBP vaccine was generated by

- mixing 1.43ml CBP-Bet v 1F1 and 1.25 ml CBP-Bet v 1F2 and
- diluting this mixture with sterile 0.9% NaCl to a total antigen concentration of 0.1mg F1+F2/ml

The vaccine was filled into 1.5mL silanized glass-vials and stored at 4°C.

Immunization of mice:

Immunization protocol: Groups of BALB/c mice (n=5) were immunized subcutaneously with the F1+F2-CBP vaccine. The immunization was done with doses containing 10µg antigen per mouse on days 1 and 28, and with CBP without antigen in NaCl as control (Pla-CBP). Blood samples were taken from the tail vein on day 0 (preimmune serum), day 27 (IS1), and day 55 (IS2).

Results: The CBP-coupled Bet v 1-derivatives induce a Bet v 1-specific IgG1 antibody response in BALB/c mice.

In order to study the development of a Bet v 1-specific antibody response upon immunization with the F1+F2-CBP vaccine blood samples were taken before and after immunization according to the immunization protocol and analyzed for Bet v 1-specific IgG1 antibodies by ELISA as described previously (2). All mice, which were immunized with F1+F2-CBP developed a Bet v 1-specific IgG1 response, which could not be detected in the control group immunized with the same amount of CBP alone (Pla-CBP).

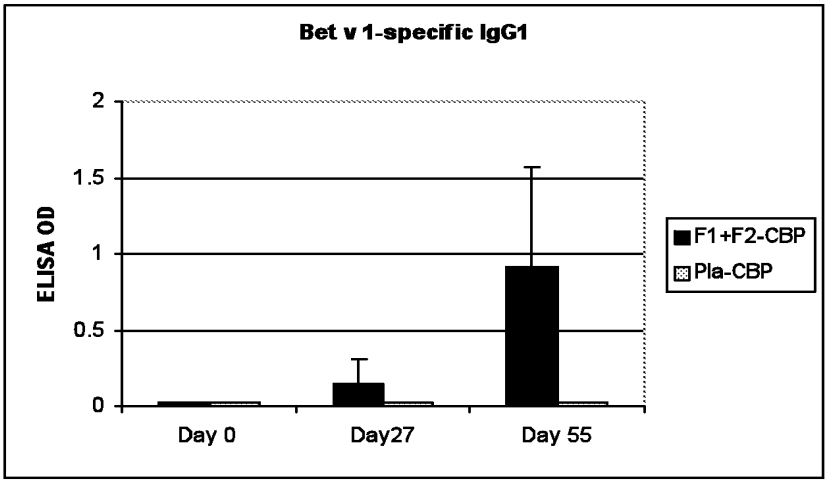

Figure 1. Development of a Bet v 1-specific antibody response in BALB/c mice using CBP as an adjuvant. Mean serum levels of Bet v 1-specific IgG1 antibodies of each mouse group measured by ELISA (ELISA OD y-axis) are shown.

Local tolerance: Injection of F1+F2-CBP induces less granulomatous reactions at the injection site if compared to injection of an Alum-adsorbed mixture of Bet v 1F1 + F2

The local tolerance of the CBP adjuvanted birch pollen allergy vaccine was investigated and compared to a vaccine containing the same antigen but Alum as an adjuvant.

Alum adsorbed F1+F2 was prepared by adsorbing an equimolar mixture of sterile-filtered Bet v 1aF1 and Bet v 1aF2 to sterile Aluminium hydroxide (Brenntag) using sterile 0.9% NaCl (Fresenius) for dilution.

Test for local tolerance: A group of 5 female mice received repeated doses (20 µg antigen) of both F1+F2-CBP and Alum adsorbed F1+F2 over a period of 23 weeks. Local tolerance was monitored by examination of the injection sides. In contrast to Alum adsorbed F1+F2, no

eczematous or granulomatous alterations were found by histopathological analysis of the skin in the case of F1+F2-CBP, emphasizing the good local tolerance of the CBP adjuvant.

References:

1. Vrtala, S., K. Hirtenlehner, L. Vangelista, A. Pastore, H. G. Eichler, W. R. Sperr, P. Valent, C. Ebner, D. Kraft, and R. Valenta. 1997. Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments: candidates for a novel form of specific immunotherapy. J Clin Invest 99: 1673-1681.

2. Linhart B, Bigenzahn S, Hartl A, Lupinek C, Thalhamer J, Valenta R, Wekerle T. Costimulation blockade inhibits allergic sensitization but does not affect established allergy in a murine model of grass pollen allergy. J Immunol. 2007; 178:3924-31.